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Cell cycle regulation in hematopoietic stem cells

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Hematopoietic stem cells (HSCs) give rise to all lineages of blood cells. Because HSCs must persist for a lifetime, the balance between their proliferation and quiescence is carefully regulated to ensure blood homeostasis while limiting cellular damage. Cell cycle regulation therefore plays a critical role in controlling HSC function during both fetal life and in the adult. The cell cycle activity of HSCs is carefully modulated by a complex interplay between cell-intrinsic mechanisms and cell-extrinsic factors produced by the microenvironment. This fine-tuned regulatory network may become altered with age, leading to aberrant HSC cell cycle regulation, degraded HSC function, and hematological malignancy.

Introduction

Hematopoiesis is the lifelong process by which all the cells of the blood system are produced in a hierarchical manner from a small population of hematopoietic stem cells (HSCs), which reside in the bone marrow (BM) cavity in adult mammals (Orkin and Zon 2008). HSCs give rise to progenitor cells that become increasingly lineage restricted and ultimately differentiate into all lineages of mature blood cells. As HSCs continually replenish cells that are lost or turned over, they must self-renew to maintain themselves over the lifetime of the organism. HSC self-renewal is experimentally defined as the capacity for long-term reconstitution of all blood lineages upon transplantation into a recipient (Ema et al., 2006). However, the capacity to self-renew is by itself insufficient for lifelong maintenance of a functional HSC compartment, as the accumulation of damage in such long-lived cells can result in dysfunctional hematopoiesis including BM failure or leukemic transformation (Lane and Gilliland 2010). Adult HSCs reside in specialized microenvironments, known collectively as the BM niche (Schofield 1978; Wilson and Trumpp 2006), where they are maintained in a quiescent, or dormant, state. It is believed that quiescence contributes to HSC longevity and function, perhaps in part by minimizing stresses due to cellular respiration and genome replication (Eliasson and Jönsson 2010).

In this review, we will focus on mouse hematopoiesis and explore the balance between HSC quiescence and proliferation, and how these two processes are regulated by intrinsic and extrinsic factors. We will also address the effects of aging on the mechanisms of HSC proliferation and quiescence, and the consequences of aging on HSC function and leukemic transformation.

Developmental origin of HSCs

Although HSCs reside in the BM in adults, this is merely the endpoint of an otherwise nomadic journey during embryogenesis. Moreover, the quiescent state of HSCs in the adult BM is reached only after a period of active cell cycling and proliferation to generate the blood system during fetal life (Bowie et al., 2006). Hematopoiesis in the embryo is considered to occur in successive waves, with the initial "primitive" wave geared toward the rapid production of red blood cells for oxygen transport but with little HSC activity; the second, or "definitive" wave, is characterized by the generation of all lineages of blood cells and the production of the first engrafting HSCs. Primitive hematopoiesis occurs as early as day E7.5 in the yolk sac blood islands (Palis et al., 1999; Medvinsky et al., 2011). The definitive wave of hematopoiesis, on the other hand, occurs in parallel in several tissues over a more protracted period of time. Definitive HSCs are found in the aorta-gonad-mesonephros (AGM) region and the placenta by E8.5 and E10, respectively, as well as in the yolk sac (Medvinsky and Dzierzak 1996; Gekas et al., 2005; Samokhvalov et al., 2007). Subsequently, HSCs from one or more of these sites expand in the fetal liver during the remainder of embryonic life, while their production by the AGM and placenta become extinguished (Medvinsky et al., 2011).

By E17.5 and through the first two weeks of postnatal life, HSCs leave the liver to colonize the bones via an active recruitment mechanism involving the CXCL12/SDF-1 chemokine receptor CXCR4 (Ma et al., 1998), which regulates HSC homing and engraftment in the nascent BM environment by activating the guanine nucleotide exchange factor Vav1, which in turn regulates the GTPases Rac and Cdc42 (Cancelas et al., 2005;

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Abbreviations used in this paper: BM, bone marrow; CKI, Cdk inhibitor; FoxO, forkhead box O; HSC, hematopoietic stem cell; MPN, myeloproliferative neoplasm; mTOR, mammalian target of rapamycin; Rb, retinoblastoma; SCF, stem cell factor; TPO, thrombopoietin.

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	Fetal HSC	Adult HSC	Aged HSC
Location	AGM Placenta Fetal Liver	Bone Marrow	Bone Marrow
Percent cycling	~100% per 24h	Active HSCs: 5.3-11.1% per 24h Dormant HSCs: 0.8-1.8% per 24h	~5% per 52h
Percent quiescent	0.02%	90-95%	~95%
Frequency of cell cycle entry	~1 per 24h	Active HSCs: ~1 per 36 days Dormant HSCs: ~1 per 145+ days	ND
Cell cycle transit time	14h (~10.5h in G_1)	14h (~10.5h in G_1)	ND
Output	Primarily erythroid & myeloid lineages	Balanced production of all blood lineages	Loss of lymphoid and erythroid potential
Physiological context of HSC activity	Blood development	Homeostatic blood production	Immunosenescence, anemia and myeloid leukemia

Figure 1. **Distinct cell cycle activity in fetal, adult, and old HSCs.** HSC cell cycle activity and cellular output is highly dynamic throughout the lifetime of an organism. During fetal life (left column), HSCs display a high level of cell cycle activity, as their primary role at this stage is the genesis of the nascent blood system. During adult life (center column), HSCs reside in the BM and enter a predominantly quiescent (G_0) state, generating a balanced myeloid, erythroid, and lymphoid output to maintain blood homeostasis. As individuals age (right column), HSCs appear to remain predominantly quiescent, but their function begins to degrade (dotted outlines), resulting in the loss of erythroid and lymphoid output and bias toward the myeloid lineage. AGM: aorta-gonad-mesonephros; ND: not determined. See text for detailed discussion and references.

Sanchez-Aguilera et al., 2011). Other factors also contribute to HSC localization to the BM either in conjunction with CXCR4, such as prostaglandin E2 (PGE2) and the neuronal guidance protein Robo4 (Hoggatt et al., 2009; Smith-Berdan et al., 2011), or independently from CXCR4 like c-Kit, the calcium-sensing receptor (CaR), and the transcription factor Egr1 (Christensen et al., 2004; Adams et al., 2006; Min et al., 2008). Thereafter, HSCs remain anchored in the BM niche by complex integrin-dependent mechanisms (Scott et al., 2003; Forsberg and Smith-Berdan 2009), though small numbers of HSCs will periodically migrate from the BM into the circulation and back for short periods of time under homeostatic conditions, perhaps as a form of immunosurveillance (Massberg et al., 2007; Bhattacharya et al., 2009). Taken together, these data underscore the dynamic nature of hematopoietic development from embryogenesis through adulthood.

Distinct cell cycle activities in fetal and adult HSCs

The cell cycle activity of HSCs over the lifetime of an organism is equally dynamic, and reflects the needs of the organism at different developmental stages. During fetal life, the central function of HSCs is to rapidly generate homeostatic levels of blood cells for oxygen transport and immune system development in the growing organism. In line with this role, between 95 and 100% of HSCs are actively cycling in the mouse fetal liver with a cell cycle transit time between 10–14 h (Fig. 1; Bowie et al., 2006; Nygren et al., 2006).

Although HSC residence in the BM during adulthood is often associated with quiescence, HSCs do not appear to become quiescent immediately upon seeding the BM, as all HSC activity remains confined to the fraction of actively cycling lineage-negative (Lin^-) BM cells in 3-wk-old weanling mice (Bowie et al., 2006). Remarkably, the BM HSC population rapidly switches to a quiescent state by 4 wk of age, with only ~5% of total HSCs actively in the cell cycle (defined as S, G_2, or M phases) thereafter through adult life (Cheshier et al., 1999; Bowie et al., 2006; Kiel et al., 2007) (Fig. 1). This abrupt change in HSC proliferation activity suggests that HSC quiescence is not solely linked to their localization in the BM cavity, but may reflect feedback mechanisms informing HSCs that blood cell formation has reached homeostatic levels, or that the development of the BM niche has been completed. Transition from active cell cycling in fetal HSCs to quiescence in adult HSCs is also associated with changes in gene expression programs, including a marked reduction in expression of *Sox17*, a transcription factor required for the maintenance of fetal but not adult hematopoiesis (Kim et al., 2007).

Interestingly, adult HSCs are not uniformly dormant. In vivo experiments assessing cell cycle activity (by measuring retention of BrdU or histone 2B (H2B)-GFP expression pulses) in mature HSCs suggest a notable heterogeneity in the degree to which HSCs are quiescent (Wilson et al., 2008; Foudi et al., 2009). These studies propose the subfractionation of the HSC compartment into "dormant" and "activated" phenotypes with distinct rates of cell cycle entry, comprising ~5–10% and 90–95% of the HSC pool, respectively (Fig. 1). Dormant HSCs are computed to divide only once every 145 d or more, and appear to be enriched for long-term reconstitution potential. This small population of cells may represent a reservoir of HSC activity kept aside in the adult BM to be called upon only by severe hematopoietic injury, thus ensuring the maintenance of blood homeostasis. However, recent work in human blood cells indicates that human HSCs enter the cell cycle on average once every 40 wk (Catlin et al., 2011). Although this finding underscores the considerable physiological difference between humans and rodents that must be kept in mind when interpreting studies performed in the mouse, it also appears to support the hypothesis that limiting cell cycle activity is critical to lifelong HSC maintenance.

Despite the great difference in the frequency by which fetal and adult HSCs divide, once in the cell cycle, they transit through it at the same slow rates compared with their more differentiated progenitor cells due to an extended passage through the G_1 phase of the cell cycle (Nygren et al., 2006). Thus, the decision regarding whether HSCs enter the cell cycle, as opposed to how they progress through it, appears to be one of the essential differences between fetal and adult hematopoiesis. Moreover, disruption of HSC quiescence leads to defects in HSC self-renewal and often results in HSC exhaustion (Orford and Scadden 2008), hence underscoring the critical importance

Table I. **Relevant genetic mouse models that have contributed to the understanding of HSC cell cycle regulation**

Category	Gene(s)	Genetic model	HSC cell cycle activity	Developmental stage	References
Cell-intrinsic mechanisms					
Cell cycle regulators	*pRb/p107/p130*	*Mx1-Cre* conditional deletion	Increased	Adult	Viatour et al., 2008
	CyclinD1/D2/D3	Triple knockout	Decreased	Fetal	Kozar et al., 2004
	Cdk4/6	Double knockout	Decreased	Fetal	Malumbres et al., 2004
	$p21^{Cip1}$	Knockout	Decreased or unchanged	Adult	Cheng et al., 2000a; van Os et al., 2007; Foudi et al., 2009
	$p57^{Kip2}$	*Mx1-Cre* conditional deletion	Increased	Adult	Matsumoto et al., 2011
	$p27^{Kip1}/p57^{Kip2}$	Double knockout; fetal liver HSC transplant	Increased	Fetal, Adult	Zou et al., 2011
	$p18^{ink4c}$	Knockout	Increased	Adult	Yuan et al 2004
Transcription factors	*p53*	Knockout	Increased	Adult	Liu et al., 2009
	p53	Hypermorphic allele	Decreased	Old	Dumble et al., 2007
	junB	*More-Cre* conditional deletion	Increased	Adult	Santaguida et al., 2009
PI3K pathway	*Pten*	*Mx1-Cre* conditional deletion	Increased	Adult	Yilmaz et al., 2006; Zhang et al., 2006
	Tsc1	Knockout	Increased	Adult	C. Chen et al., 2008
	Akt1/2	Double knockout	Decreased	Adult	Juntilla et al., 2010
	myr-Akt	Retroviral expression	Increased	Adult	Kharas and Gritsman 2010
	Foxo1/3/4	Triple knockout	Increased	Adult	Tothova et al., 2007
	Foxo3	Knockout	Increased	Adult	Miyamoto et al., 2007
Cell-extrinsic mechanisms					
Environmental factors	*Tpo*	Knockout	Increased	Fetal, Adult	Qian et al., 2007
	Ang-1	Retroviral expression	Decreased	Adult	Arai et al., 2004
	Kit	Hypomorphic allele	Increased	Fetal, Adult	Thorén et al., 2008
	Cxcr4	$Rosa^{Cre\text{-}Ert2}$ conditional deletion	Increased	Adult	Nie et al., 2008
Developmental pathways	*Dkk*	*Col1α2.3*-driven transgene	Increased	Adult	Fleming et al., 2008
	Gli1	Knockout	Decreased	Adult	Merchant et al., 2010

of a constitutively low level of cell cycle activity for proper function of the blood system during adult life.

Together, these findings underscore the requirement for a complex network of regulatory mechanisms in enforcing the balance between HSC quiescence and proliferation, and the proper maintenance of blood homeostasis in the adult organism. In the following sections, we will discuss the current understanding of such intrinsic and extrinsic regulatory mechanisms in adult HSCs and highlight the relevant genetic mouse models that have contributed to this understanding (Table I).

Cell-intrinsic mechanisms regulating HSC quiescence

Numerous factors regulate the cell cycle status within a cell; however, they can largely be reduced to the competing actions of Cdks, which drive cell cycle progression, and Cdk inhibitors (CKIs), which blunt progression through the cell cycle (Morgan 1997). A large body of studies has begun to unravel the molecular wiring within adult HSCs that mediate their continued maintenance in a quiescent, or G_0, state, while allowing for their rapid entry into the cell cycle to respond to hematopoietic demand (Fig. 2).

Rb Family. The retinoblastoma (Rb) family of transcriptional repressors, including the pRb, p107, and p130 proteins, restricts cell cycle entry by repressing E2F gene transcription of positive cell cycle regulators, which include E-type cyclins. In their hypophosphorylated state, Rb family members restrict entry through G_1 phase. However, upon phosphorylation by the cyclin D–Cdk4/6 complex, Rb family members are partially inactivated and permit cell cycle progression through G_1. Subsequent phosphorylation by cyclin E–Cdk2 further inactivates Rb-mediated inhibition of E2F, resulting in G_1 exit and entry into S phase. A firm role for Rb family members in the control of HSC quiescence was not established until recently, as considerable functional redundancy exists within this family and as all members are expressed, albeit at different levels, in HSCs (Passegué et al., 2005). For instance, no hematopoietic phenotype was observed in *p130*-deficient mice (Cobrinik et al., 1996), and *p107* deletion resulted in only a mild myeloid hyperplasia (LeCouter et al., 1998). Removal of *pRb* had also no effect on HSC self-renewal as assessed by serial transplantation (Walkley and Orkin 2006), and although *pRb*-deficient mice exhibited myeloid expansion, this was shown to be a non-cell autonomous effect (Walkley and Orkin 2006; Walkley et al., 2007). Strikingly, however, conditional deletion of all three Rb family members in

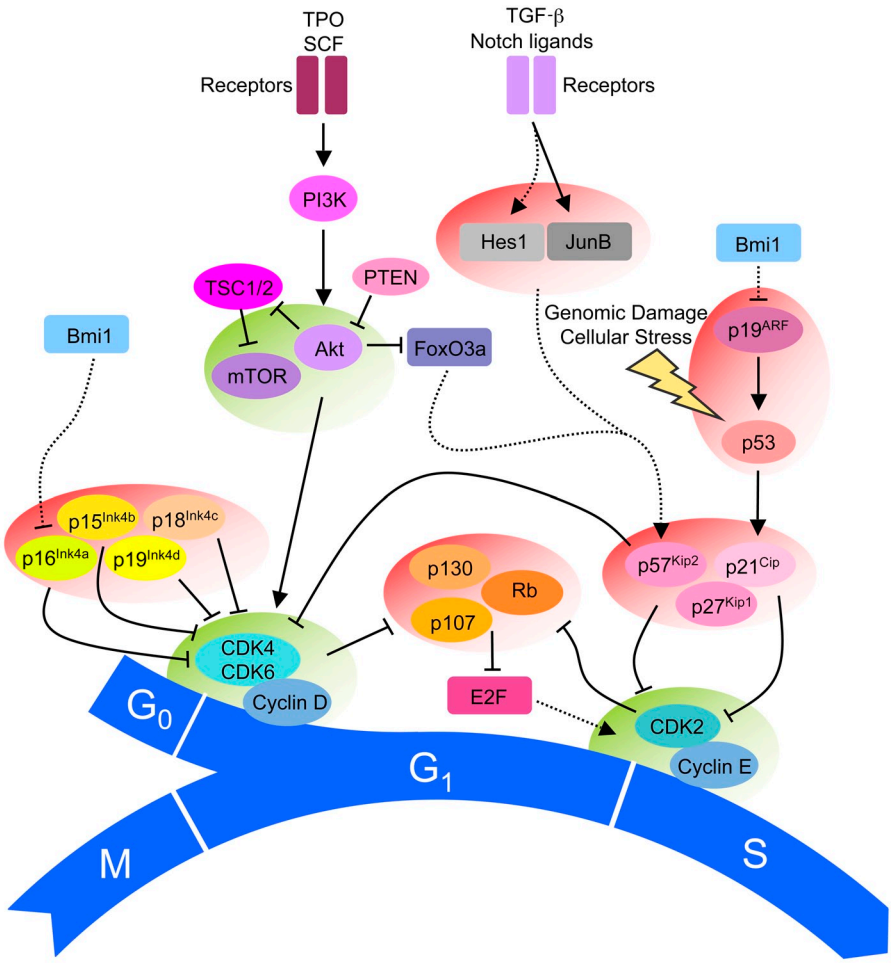

Figure 2. **HSC cell cycle entry is regulated by a complex network of cell-intrinsic and cell-extrinsic factors.** The entry of quiescent HSCs from G_0 into the G_1 phase of the cell cycle is governed primarily via competing activating and inhibitory mechanisms that regulate the activity of cyclin–Cdk complexes. The PI3K/Akt/mTOR pathway, which is activated in response to numerous extrinsic signals, is considered a central activator of HSC cell cycle activity, primarily via activation of the cyclin D–Cdk4/6 complex. This pathway is heavily regulated, primarily by PTEN and TSC1/2. Moreover, the Ink4 CKI family inhibits cyclin D–Cdk4/6 activity, and CIP/KIP family CKIs are also capable of inhibiting Cdk4 activity. Progression from the G_1 to the S phase of the cell cycle is regulated by Cyclin E–Cdk2. This complex is regulated via the CIP/KIP family of Cdk inhibitors, as well as by the Rb family. Expression of CIP/KIP family members is in turn regulated by transcription factors such as Hes1, JunB, and FoxO3a, which are activated by extrinsic growth-repressive signals. Furthermore, HSC cell cycle activity is subject to regulation via p53, either in response to cellular damage or $p19^{ARF}$ activity. Solid arrows indicate direct activation/inhibition events, dashed arrows indicate transcriptional regulation events. Functionally related groups of cell cycle activators are shaded in green; functionally related groups of cell cycle inhibitors are shaded in red.

adult mice resulted in a robust cell-intrinsic myeloproliferation phenotype leading to the death of the animals by 1–3 mo after gene inactivation (Viatour et al., 2008). This was accompanied by an increase in both HSC proliferation and absolute cell numbers, and by severe defects in HSC self-renewal as BM from mice deficient in all three *Rb* family genes had grossly impaired reconstitution after transplantation (Viatour et al., 2008). Taken together, these findings indicate that Rb family members play critical, albeit overlapping roles in the regulation of HSC quiescence and continued self-renewal activity.

D-cyclins and Cdk4/6. Cyclins and Cdks act upstream of the Rb family members to mediate cell cycle entry and progression. As the regulation of HSC quiescence fundamentally involves controlling whether HSCs enter the G_1 phase of the cell cycle or remain in G_0, the activity of the cyclin D–Cdk4/6 complex, which controls progression through G_1 in response to mitogenic signals, is likely a central determinant of HSC cell cycle activity. The D-cyclin family includes cyclin D1 (*Ccnd1*), cyclin D2 (*Ccnd2*), and cyclin D3 (*Ccnd3*), which are all expressed, albeit at different levels, in HSCs (Passegué et al., 2005). Similar to the Rb gene family, mice deficient for a single D-cyclin, or for only one of the two associated Cdks, have minimal hematopoietic defects, hence illustrating the considerable functional redundancy protecting this complex (Fantl et al., 1995; Malumbres et al., 2004). However, mice deficient in all three D-cyclins die during late embryogenesis due to heart defects and hematopoietic failure with significant reduction in peripheral red blood cell numbers (Kozar et al., 2004). Furthermore, *D1/2/3-cyclins*$^{-/-}$ mice have lower numbers of HSCs and progenitor populations in the fetal liver, with decreased frequency of HSCs in S and G_2-M stages of the cell cycle. Fetal liver cells derived from *D1/2/3-cyclins*$^{-/-}$ mice are also unable to provide short-term reconstitution of irradiated recipient mice (Kozar et al., 2004). *Cdk4/6*$^{-/-}$ mice also display late embryonic lethality accompanied by a defect in fetal hematopoiesis very similar to the phenotypes observed in *D1/2/3-cyclins*$^{-/-}$ mice, including severely decreased numbers of proliferating erythroid progenitors in the fetal liver and of red blood cells circulating in the peripheral blood (Malumbres et al., 2004). These findings further underscore the essential requirement for active HSC proliferation during fetal blood development and stress the importance of the cyclin D–Cdk4/6 complex in fetal HSC cell cycle progression. However, it is yet to be established whether there is a different requirement for the cyclin D–Cdk4/6 complex in maintaining the proliferation and functionality of adult HSCs.

Ink4 family. The Ink4 family includes the CKIs $p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$, and $p19^{Ink4d}$, and a functionally distinct protein, $p19^{ARF}$ that is encoded by an alternate reading frame within the *Ink4a* locus, which also encodes $p16^{Ink4a}$ (Sherr 2001). The Ink4

proteins all function as antagonists of the cyclin D–Cdk4/6 complex, thereby blocking phosphorylation of Rb family members and subsequent entry into S phase. $p19^{ARF}$, on the other hand, is not a CKI and instead positively regulates p53 (see CIP/KIP family section; Sherr 2001). Deletion of both $p16^{Ink4a}$ and $p19^{ARF}$ through the disruption of the entire *Ink4a* locus has minimal consequences for HSC activity, consistent with the reported low or absent expression of these factors in HSCs (Passegué et al., 2005). Their limited expression is likely the result of transcriptional repression by Bmi1, a polycomb group family member and chromatin remodeler that is expressed preferentially in HSCs and actively represses the Ink4a locus (Lessard and Sauvageau 2003; Park et al., 2003). *Bmi1* deficiency is lethal in adult mice due to hematopoietic failure caused by a progressive depletion of HSCs (Lessard and Sauvageau 2003; Park et al., 2003). This result implies that the resulting increase in $p16^{Ink4a}$ and $p19^{ARF}$ expression caused by *Bmi1* removal may completely inhibit the infrequent cell cycle entry of adult HSCs, which is essential for HSC self-renewal and maintenance of blood homeostasis. The fact that combined deletion of $p16^{Ink4a}$ and $p19^{ARF}$ rescues the majority of the hematopoietic phenotypes in *Bmi1*-deficient mice supports this notion and indicates a critical role for Bmi1 in restraining $p16^{Ink4a}$ and $p19^{ARF}$ expression in adult HSCs (Oguro et al., 2006). In contrast, deletion of $p18^{Ink4c}$ directly results in increased numbers of actively cycling HSCs without impairment of HSC self-renewal activity, as $p18^{Ink4c}$-deficient cells confer enhanced reconstitution to lethally irradiated recipients (Yuan et al., 2004). Notably, $p18^{Ink4c}$ expression is highest in quiescent HSCs, and is significantly decreased in actively cycling fetal HSCs relative to adult HSCs, consistent with its role as a brake for HSC proliferation (Passegué et al., 2005; Bowie et al., 2007). This result may also provide some molecular basis for the difference in cell cycle activity between fetal and adult HSCs. Collectively, these data indicate that several of the Ink4 family members are differentially regulated in adult HSCs to maintain the proper balance between quiescence and proliferation, with $p18^{Ink4c}$ directly acting to restrict cell cycle entry, and Bmi1 preventing expression of $p16^{Ink4a}$ and $p19^{ARF}$.

CIP/KIP family. The CIP/KIP family includes the CKIs $p21^{Cip}$, $p27^{Kip1}$, and $p57^{Kip2}$, which also restrain entry into S phase by inhibiting the activity of the cyclin E–Cdk2 complex. $p21^{Cip}$ is expressed at somewhat greater levels in adult HSCs relative to their differentiated progeny or to fetal HSCs (Passegué et al., 2005; Bowie et al., 2007). Although a role for $p21^{Cip}$ in regulating HSC quiescence had initially been suggested (Cheng et al., 2000a), more recent reports using different mouse backgrounds and/or methodologies suggest that the function of $p21^{Cip}$ in regulating HSC cell cycle activity may be restricted to periods of stress rather than during homeostasis (Cheng et al., 2000a; van Os et al., 2007; Foudi et al., 2009). Aside from $p21^{Cip}$, analysis of $p27^{Kip1}$-deficient mice suggests that $p27^{Kip1}$ deficiency alone has a limited impact on HSC function and instead appears to affect the cell cycle activity of more committed progenitor populations (Cheng et al., 2000b). Interestingly, although earlier studies detected no overt hematopoietic defects in $p57^{Kip2}$-deficient mice (Yan et al., 1997; Zhang et al., 1997), more recent work using hematopoietic-specific deletion of $p57^{Kip2}$ in adult mice suggests that $p57^{Kip2}$ is in fact critical for maintaining HSC quiescence (Matsumoto et al., 2011). In addition, the defective quiescence phenotype of $p57^{Kip2}$-deficient HSCs is exacerbated by concomitant deletion of either $p21^{Cip}$ *or* $p27^{Kip1}$, and $p27^{Kip1}$ overexpression is able to compensate for the loss of $p57^{Kip2}$ (Matsumoto et al., 2011; Zou et al., 2011). Taken together, these studies reveal $p57^{Kip2}$ as a critical regulator of HSC quiescence in adult mice, and suggest some degree of functional redundancies among the CIP/KIP family of CKIs. Interestingly, growth-repressive signals such as TGF-β directly target the $p21^{Cip}$ and $p57^{Kip2}$ genes, suggesting a role for the BM niche in regulating expression of these CKIs in HSCs (Scandura et al., 2004). Interestingly, p53, a master transcriptional regulator that induces the transcription of $p21^{CIP}$ in addition to a plethora of other genes upon cellular insult, also regulates HSC quiescence (Asai et al., 2011). $p53^{-/-}$ HSCs have increased BrdU incorporation and decreased frequency of G_0 cells (Liu et al., 2009). Although *p53* deletion increases the numbers of phenotypic HSCs, transplantation experiments demonstrate that $p53^{-/-}$ HSCs actually have decreased functionality, suggesting that p53 positively regulates self-renewal, perhaps by restraining HSC activity (TeKippe et al., 2003; J. Chen et al., 2008). However, this appears to be independent of $p21^{Cip}$, and may instead be due to the transcription of other p53 targets that negatively regulate HSC proliferation, particularly *Necdin* and *Gfi-1* (Liu et al., 2009).

PI3K signaling pathway. The phosphatidylinositol-3 kinase (PI3K) signaling pathway integrates numerous upstream signals from activated cytokine receptors and other mitogenic stimuli to drive cell proliferation, growth, and survival. Downstream targets of the PI3K pathway include the threonine/serine kinase Akt, which can both lead to the activation of mammalian target of rapamycin (mTOR) and the suppression of the forkhead box O (FoxO) family of transcription factors. This pathway is restrained through the action of the tumor suppressor phosphatase and tensin homologue (PTEN). Specifically, PI3K signaling may accelerate cell proliferation by stabilizing D-type cyclins and inhibiting FoxO-dependent transcription of $p21^{Cip}$ and $p27^{Kip1}$ (Massagué 2004). Attenuation of signaling through the PI3K pathway is essential to preserve HSC quiescence and long-term self-renewal (Warr et al., 2011). Conditional deletion of *Pten* in the hematopoietic system results in an aggressive and deadly early-onset myeloproliferative neoplasm (MPN), which is accompanied by a threefold increase in the frequency of cycling HSCs (Yilmaz et al., 2006; Zhang et al., 2006). $Pten^{-/-}$ HSCs also show defective self-renewal activity as they are unable to provide long-term engraftment in transplanted mice, which results in the depletion of the HSC pool in *Pten*-deficient mice (Yilmaz et al., 2006; Zhang et al., 2006). Increased HSC cycling and defective self-renewal activity are also observed in a myristoylated *Akt* retroviral transplantation model, which has constitutive Akt activity (Kharas and Gritsman 2010), and in mice lacking the tumor suppressor *Tsc1*, which has unchecked mTOR activity (C. Chen et al., 2008). Furthermore, rapamycin, a pharmacological inhibitor of mTOR, is able to reverse many of the phenotypes associated with *Pten* deficiency, including the increased proliferation of HSCs (Yilmaz et al., 2006). These results

suggest that PTEN normally restrains HSC entry into the cell cycle entry, at least in part, through inhibition of downstream mTOR signaling. However, as discussed previously, constitutive levels of Akt also repress the activity of FoxO family members. Interestingly, *FoxO3*$^{-/-}$ and *FoxO1/3/4*$^{-/-}$ mice display increased HSC cell cycle activity accompanied by increased levels of reactive oxygen species (ROS), which can be reversed after the administration of the ROS scavenger *N*-acetylcysteine (NAC; Miyamoto et al., 2007; Tothova et al., 2007). In contrast, loss of PI3K signaling caused by deletion of *Akt1/2* results in an increased proportion of HSCs in G_0/G_1 phase and decreased HSC proliferation, which can be rescued in vitro by pharmacologically increasing cellular ROS levels (Juntilla et al., 2010). Although cellular oxygen levels and HSC proliferation are thought to be directly associated (Ito et al., 2006; Eliasson and Jönsson 2010; Takubo et al., 2010), a direct demonstration of the function of ROS in controlling HSC cell cycle in vivo remains to be provided. Collectively, these studies suggest that HSC quiescence is preserved at least in part by limiting activation of the PI3K signaling pathway, which may maintain high levels of FoxO-dependent expression of CKIs and minimize ROS production, thereby blocking HSC cell cycle progression.

Cell-extrinsic mechanisms regulating HSC quiescence

Although quiescence is essential for the self-renewal of adult HSCs, they must nonetheless retain the capacity to proliferate rapidly, albeit transiently, in response to extrinsic cues that signal injury or infection. Evidence that cell-extrinsic signals regulate the cell-intrinsic mechanisms governing HSC quiescence can be inferred from the observation that HSCs enter the cell cycle upon pharmacological mobilization in vivo or culture ex vivo (Passegué et al., 2005), and from experimental evidence indicating that osteoblasts and other cellular components of the BM niche influence HSC cell cycle status (Wilson et al., 2007).

Part of this mechanism proceeds via a series of conserved developmental pathways, including TGF-β, Wnts, Notch ligands, and the Hedgehog (Hh) pathway, which are all essential for embryonic development and fetal hematopoiesis. Deletion of *TGF-β1* or *Indian hedgehog* (*Ihh*) lead to defects in fetal erythropoiesis resulting in embryonic lethality (Dickson et al., 1995; Cridland et al., 2009), whereas *Wnt3a*-deficient mice show depressed numbers of HSCs in the fetal liver (Luis et al., 2009). However, conclusive in vivo evidence for their role in adult HSC cell cycle regulation has been difficult to generate, as *Mx-Cre*–driven conditional deletion of Notch, Wnt, and Hh signaling components in adult mice do not reveal clear hematopoietic defects or alterations in HSC cell cycle activity (Koch et al., 2008; Maillard et al., 2008; Gao et al., 2009). This suggests that they are either not absolutely critical in the context of adult hematopoiesis, or that significant functional redundancy exists between these and other niche signals. Additional work will be required to disentangle the complex direct and indirect effects of these developmental pathways on adult HSCs in vivo, as well as the relevant receptor–ligand interactions on both HSCs and/or BM niche cells that mediate them. Furthermore, it should be emphasized that these pathways may also act as critical regulators of HSC cell cycle activity in specific hematological contexts that have not yet been well studied, such as stress or disease (Zhao et al., 2007, 2009).

A wide range of environmental factors, including cytokines, growth factors, and other mediators produced by circulating immune cells and cells comprising the BM niche, also modulate HSC quiescence. In contrast to the previously discussed developmental pathways, these environmental factors, which include the chemokine CXCL12/SDF-1, stem cell factor (SCF), angiopoietin-1 (Ang-1), and thrombopoietin (TPO), are dispensable for fetal hematopoiesis, but are essential to regulate HSC quiescence in adult organisms (Wilson et al., 2009). Mice deficient in *Cxcr4* (SDF-1 receptor), *Tpo, Tie-2* (Ang-1 receptor), or carrying a hypomorphic allele of *Kit* (SCF receptor), all exhibit normal fetal hematopoiesis followed by increased cell cycle activity and progressive loss of the HSC compartment during adult life (Nagasawa et al., 1996; Puri and Bernstein 2003; Arai et al., 2004; Qian et al., 2007; Nie et al., 2008; Thorén et al., 2008). Collectively, these results highlight the importance of cell-extrinsic mechanisms in regulating HSC function, and further underscore the importance of quiescence for the maintenance of the HSC compartment during adulthood.

A body of evidence suggests that the regulation of lipid raft clustering on the surface of HSCs may be a critical determinant of HSC quiescence by dictating the level of Akt activation induced by cytokine receptors. Quiescent HSCs show minimal amounts of lipid raft clustering, while actively proliferating hematopoietic progenitor cells have high levels of clustering and Akt pathway activation (Yamazaki et al., 2006, 2007). In addition, SCF or TPO stimulation of HSCs in in vitro culture conditions lead to lipid raft clustering, rapid Akt phosphorylation, and exclusion of FoxO3a from the nucleus, resulting in decreased expression of $p21^{Cip}$ and $p57^{Kip2}$ and reentry of HSCs into the cell cycle (Yamazaki et al., 2006, 2007). Interestingly, TGF-β signaling suppresses lipid raft clustering in HSCs, hence limiting the activation of Akt induced by SCF or TPO stimulation to a level that likely promotes HSC survival but not proliferation (Yamazaki et al., 2009). TGF-β also directly induces expression of $p57^{Kip2}$, and prevents sequestration of cyclin D1 (Yamazaki et al., 2009; Scandura et al., 2004). Taken together, these results explain a mechanism by which TGF-β may enforce HSC quiescence, although the relationship between TGF-β and HSC quiescence has not been directly addressed in vivo due to the confounding effect of an autoimmune condition present in mice lacking TGF-β signaling components (Larsson et al., 2003). These data may also help explain why SCF and TPO are associated with HSC quiescence and not proliferation in vivo, as factors such as TGF-β that are present in the BM niche may prevent activation of Akt by these cytokines. Furthermore, TGF-β also collaborates with Notch ligands to induce expression of $p21^{Cip}$ via a mechanism involving the transcription factors junB and Hes1 (Yu et al., 2006; Santaguida et al., 2009). Mice deficient in the transcription factor Smad4, which interacts with TGF-βR–activated Smad2/3, express lower levels of *Notch1* but the effect of *Smad4* deficiency on HSC cell cycle activity has not yet been directly studied (Karlsson et al., 2007). These findings suggest that TGF-β and Notch may function in an interdependent manner to regulate HSC cell cycle activity. In addition, the induction of $p21^{Cip}$ and $p57^{Kip2}$ expression is also

regulated by a number of other BM niche-associated factors, including TPO, Wnt, and SDF-1 (Qian et al., 2007; Fleming et al., 2008; Nie et al., 2008). Collectively, these data indicate that multiple signaling mechanisms activated by developmental pathways and environmental factors may converge upon the regulation of CKI expression, which in turn might contribute to the prolonged passage of HSCs through the G_1 phase of the cell cycle.

Regulation of cyclin expression forms another convergence point by which extrinsic signals regulate the internal machinery governing quiescence in adult HSCs. Mice deficient in *Cxcr4* have increased levels of cyclin D1, which suggests that the CXCR4–SDF-1 pathway impacts on the activity of the cyclin D–Cdk4/6 complex and G_1 phase progression in HSCs (Nie et al., 2008). Notably, TGF-β and Notch ligands up-regulate CXCR4, and this may account for some of their quiescence-enforcing activity (Franitza et al., 2002). On the other hand, activation of the Wnt and Hh pathways is associated with increased expression of cyclin D1 in HSCs, although Wnt signaling is typically associated with maintenance of HSC quiescence and self-renewal activity (Fleming et al., 2008; Merchant et al., 2010). It may be that signaling through these pathways helps to maintain basal expression of cyclin D1 in quiescent HSCs (Passegué et al., 2005). Such careful balancing of pro- and anti-proliferative factors in the BM niche could account for their role in enforcing a homeostatic level of HSC quiescence, while leaving HSCs sufficiently primed for proliferation in response to hematopoietic demand. Recent work suggests that interferons, a family of inflammatory cytokines, rapidly induce HSC cell cycle entry, and this may occur at least in part via activation of the Akt pathway (Essers et al., 2009; Baldridge et al., 2010). These results confirm that the quiescent state of HSCs is indeed quickly reversible, which is likely to be critical for effective control of stress conditions such as infection or blood loss.

Taken together, these studies demonstrate that quiescence is a dynamic condition that is orchestrated by a careful balance between collaborating and opposing extrinsic signals acting on various components of the intrinsic cell cycle machinery, which directly control HSC proliferation and self-renewal activity (Fig. 2). Further studies of the mechanisms by which these extrinsic factors regulate the proliferation of fetal vs. adult HSCs will be invaluable in understanding the regulation of the cell cycle machinery in HSCs and other stem cells as organisms reach maturity. A careful analysis of how these extrinsic factors may contribute to the heterogeneity observed in the cycling activity of HSC subpopulations should also prove highly informative (Challen et al., 2010).

Aging and the control of HSC cell cycle activity

The hematopoietic system declines with age, resulting in a diminished production of adaptive immune cells, termed immunosenescence, and an increased incidence of anemia and myeloid malignancies (Fig. 1; Beerman et al., 2010b). Although aged mice have increased numbers of phenotypic HSCs, the functional capacity of these cells is actually compromised, as evidenced by the decrease in long-term multilineage reconstitution potential of purified old HSCs transplanted into young recipient mice (Rossi et al., 2005). However, whether this is a result of a true change in self-renewal versus a clonal change in the composition of the HSC compartment is currently debated (Cho et al., 2008; Waterstrat and Van Zant 2009; Beerman et al., 2010a).

During aging, the additive effects of repeated cellular and genomic insults can become apparent, potentially damaging the integrity of the HSC pool (Blanpain et al., 2011). Indeed, mice deficient for key DNA repair pathway components exhibit diminished HSC self-renewal with age and early functional exhaustion (Nijnik et al., 2007; Rossi et al., 2007). In fact, long-lived quiescent HSCs may be particularly susceptible to the accumulation of DNA damage over time, as their cell cycle status forces them to repair DNA damage using the error-prone and mutagenic nonhomologous end joining (NHEJ) mechanism (Mohrin et al., 2010). Moreover, HSCs from aged mice exhibit increased phosphorylated γ-H2AX staining, which indicates increased occurrence of DNA double-strand breaks, and suggests that aging may contribute to the loss of HSC function in part due to genomic instability (Rossi et al., 2007). In addition to DNA damage, the short-circuiting or overload of epigenetic control mechanisms regulating HSC self-renewal may also contribute to the pathogenesis of hematological malignancies, particularly in the myeloid lineage, later in life (Rossi et al., 2008). Indeed, deletion of Ten-Eleven-Translocation-2 (Tet2), a factor thought to regulate gene silencing via methylation and which is commonly mutated in individuals with MPNs, leads to aberrant activation of myeloid differentiation gene programs in HSCs and subsequent MPN emergence in mice (Ko et al., 2011, Li et al., 2011, Moran-Crusio et al., 2011, Quivoron et al., 2011). Thus, the appropriate control of HSC cell cycle activity may therefore become particularly important in an aging organism as a means of preventing the outgrowth of damaged clones via induction of cell cycle arrest or cellular senescence.

Studies of aged mice show an overall decrease in HSC cell cycle activity, with old HSCs undergoing fewer cell divisions than young HSCs as assessed by BrdU and *N*-hydroxysuccinimidyl biotin (NHS-biotin) incorporation (Janzen et al., 2006; Nygren and Bryder 2008). These results suggest that Cdks and other activators of the cell cycle may undergo functional decline or that the activity of certain cell cycle checkpoint mechanisms such as CKIs may increase with age, hence delaying HSC entry into the cell cycle. This latest hypothesis is supported by the observations of increased $p16^{INK4a}$ levels in old HSCs and of better engraftment capacity and increased cell cycle activity in old $p16^{INK4a-/-}$ HSCs compared with old wild-type HSCs (Janzen et al., 2006). As increased expression of $p16^{Ink4a}$ is associated with aging in numerous tissues, and its expression correlates with cellular senescence (Sharpless and DePinho 2007), it is appealing to suggest that $p16^{Ink4a}$ expression in aged HSCs may result in the cellular senescence of at least a subpopulation of old HSCs. However, another group demonstrates no correlation between $p16^{Ink4a}$ expression levels and HSC aging (Attema et al., 2009). Such discrepancies may in part be due to heterogeneity among cohorts of aged mice, or the sensitivity of different methods used to detect $p16^{Ink4a}$ expression. Interestingly, Bondar and Medzhitov (2010) elegantly demonstrated that cell competition exists in

the HSC compartment and is dependent on the relative levels of p53 expression, and a balance between proliferation and senescence responses. This work shows that cells with lower levels of p53 outcompete cells with higher p53 levels for BM engraftment, resulting in growth arrest and the induction of a senescence-related gene expression program in the outcompeted cells, which includes $p16^{Ink4a}$ expression. Furthermore, HSCs from old $p53^{+/m}$ mice, which carry a hypermorphic allele of *p53*, exhibit decreased proliferation and self-renewal capacity relative to old $p53^{+/+}$ controls, whereas old $p53^{+/-}$ HSCs, which have only one functional *p53* allele, exhibit increased function compared with controls (Dumble et al., 2007). Notably, such differences are not observed in HSCs from young mice, regardless of *p53* status. Collectively, these data suggest that p53-dependent checkpoint mechanisms, perhaps including but not limited to induction of $p16^{Ink4a}$, may in part underlie the functional decline and decreased cell cycle activity of aged HSCs. This might be due to a combination of cellular senescence in some HSCs amid an expansion of other functionally compromised HSC clones in which cell cycle checkpoints have been compromised. Such a model may help explain the apparent lack of senescent HSCs observed in the expanded, but functionally impaired, HSC compartment of old mice, while at the same time accounting for the improved function of the HSC pool in aged $p16^{INK4a-/-}$ mice, where otherwise competitively disadvantaged HSC populations may still contribute to blood production.

As described previously, mouse models that lead to unchecked levels of mTOR activity result in a deregulated HSC compartment and stem cell exhaustion (Yilmaz et al., 2006; Zhang et al., 2006; C. Chen et al., 2008). Recent work also indicates that mTOR activity is increased in aged HSCs (Chen et al., 2009). Interestingly, pretreatment of aged mice with rapamycin, which inhibits mTOR activity, rescues many of the functional defects observed in old HSCs, including their decreased cell cycle activity and diminished engraftment (Chen et al., 2009). At the moment it is quite challenging to integrate this observation with the aforementioned increase in $p16^{Ink4a}$ expression exhibited by the old HSCs because increased mTOR activity results in increased HSC cycling, at least in young $Pten^{-/-}$ and $Tsc1^{-/-}$ HSCs (Yilmaz et al., 2006; C. Chen et al., 2008). However, it is plausible that in aged mice, mTOR levels do not reach a similar threshold as observed in these mutant cells, or that mTOR signaling exerts differential effects in dysfunctional old HSCs.

Conclusions and future perspectives

Hematopoiesis is a dynamic process that has evolved to generate and maintain homeostatic levels of blood cells for the lifetime of the organism. The lifelong persistence of HSCs is likely due to their acquisition of a quiescent phenotype upon maturity of the host, as well as their localization to specialized niches in the BM cavity wherein extrinsic cues regulate their cell cycle activity. Both of these features can be envisioned as strategies to limit the frequency and severity of potentially damaging replicative stresses. The aging hematopoietic system serves as a compelling model in which to examine the limitations of these protective strategies. The work reviewed here suggests that an old hematopoietic system, in which HSCs may have acquired damage, must deal with the conflicting requirements of suppressing the outgrowth of damaged and/or transformed clones, while at the same time producing enough blood cells to maintain homeostasis. The biological response to this problem appears to err on the side of caution, as evidenced by the decreased self-renewal potential and reduced cell cycle entry observed in the expanded pool of old HSCs. Although it would be tempting to explain these responses as a p53- and/or $p16^{Ink4a}$ -driven program of cellular senescence that could underlie some of the hematological insufficiencies observed in the elderly, experimental evidence is still needed to confirm this hypothesis. The high incidence of hematological malignancy observed with age suggests that the ability to arrest damaged HSCs is likely not without fundamental limitations, hence supporting the notion that expansion of functionally deficient HSC clones harboring defective cell cycle checkpoints contributes to the aging of the blood system (Kastan and Bartek 2004; Bondar and Medzhitov 2010).

Considerable work still remains to be done to fully understand how components of the regulatory networks that control cell cycle activity in young adult HSCs may become altered with age. For instance, is there a global change in the expression levels of master cell cycle regulators in old HSCs? Although microarray analyses have been performed, they do not uncover any particular changes in cell cycle machinery or upstream regulatory genes in old HSCs, though alterations in the expression of genes involved in myeloid and lymphoid differentiation and epigenetic regulation are observed (Rossi et al., 2005; Chambers et al., 2007). These results suggest that the activity, rather than the expression level, of cell cycle regulators is altered in old HSCs, which is consistent with the work showing increased mTOR activity in old HSCs (Chen et al., 2009). Furthermore, as the activation level of the cell cycle machinery is in part regulated by cell-extrinsic signals, it will be critical to understand the extent to which the extracellular environment where HSCs reside, both in the niche and systemically, is altered with age and contributes to the functional decline of the hematopoietic system.

Taken together, these findings underscore the challenges inherent in understanding the cell cycle regulation of a system as complex and dynamic as the aging hematopoietic system, as aging itself appears to alter the cellular context in which these regulatory mechanisms operate. It can be speculated that the functional decline of the blood system could be in part due to a clonal expansion of damaged HSC populations that outcompete HSCs with intact cell cycle checkpoints. Thus, experimental approaches that uncover the extent to which distinct subpopulations of HSCs and their output change over time will likely be crucial to our understanding of how the phenotypic features of aged hematopoiesis arise. Understanding how aging affects HSC biology will also provide critical insight into the pathogenesis of hematological malignancies that result from such an outgrowth of damaged HSCs. In turn, the development of targeted therapies that modulate the activity of cell cycle checkpoints in HSCs may prove efficacious in eliminating damaged HSC clones or in reducing their output. Such therapies may reestablish favorable conditions for the activation of undamaged HSCs and a restoration of effective hematopoiesis in aged individuals or patients with hematological

malignancies. Moreover, the results of studies using HSCs as a developmental model system may be broadly applicable to other tissues in which stem cells are either not as well characterized or as easily isolated. Thus, the continued elucidation of the mechanisms controlling cell cycle activity in fetal, adult, and old HSCs will have significant impact at the intersection between cancer and stem cell biology, both at the bench and in the clinic.

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